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Crop Immune Response Post–*Salmonella* Enteritidis Challenge in Eight Commercial Egg-Layer Strains and Specific-Pathogen-Free White Leghorn Chickens

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SUMMARY. The crop immune response against *Salmonella* Enteritidis (SE) challenge in eight commercial egg-layer strains (five white-egg layer and three brown-egg layer) and specific-pathogen-free (SPF) White Leghorn (WL) hens was investigated. Pre- and post-SE challenge mucosal immune responses within the crops were evaluated. Commercial layers and SPF WL hens were orally challenged with 10^8 CFU/ml SE PT13a and SE nalR PT13, respectively. Crop lavage samples were collected at weekly intervals from day 0 (pre-challenge) to day 25–27 postinfection (PI), and bacteriological examination was performed to monitor progression of SE infection. Crop lavage samples were analyzed for SE-lipopolysaccharide (LPS)–specific IgA using enzyme-linked immunosorbent assay (ELISA). H&E-stained slides of crop sections from day 34 PI and uninfected controls were assessed for lymphoid tissue via light microscopy. Lymphoid areas were graded based on morphology, size, and cellularity using a score 0 to 5 scale. The 0 to 5 (low to high) numerical values represented progressive increases in size and cellular density of lymphoid tissue. Bacterial culture results showed the highest percentage of SE-positive crop lavage samples from all hen groups at day 5–6 PI and day 11–12 PI. A progressive decline in percentage of SE-positive crop lavage samples did occur as time PI lengthened; however, at day 25–27 PI SE persisted in crop lavage samples from SPF WL hens and three commercial white-egg layer strains. A marked increase in SE-LPS-specific IgA was measured in crop lavage samples between day 0 and day 11–12 PI for all hen groups. Crop SE-LPS-specific IgA response remained elevated above day 0 baseline for the duration of the experiment. Well-defined score 3 to 5 lymphoid tissue aggregates were observed in crop tissue sections harvested at day 34 PI. Comparison of crop sections determined a 1.2–4.0 times increase in ratio of lymphoid tissue in day 34 PI SE-challenged hens *vs.* uninfected control hens.

RESUMEN. Respuesta inmune en el buche frente a un desafío con *Salmonella* Enteritidis en ocho líneas de ponedoras comerciales y en aves tipo leghorn libres de patógenos específicos.

Se investigó la respuesta inmune en el buche frente a un desafío con *Salmonella* Enteritidis en ocho líneas de ponedoras comerciales (cinco ponedoras de huevos blancos y tres ponedoras de huevos marrón) y en aves Leghorn blancas libres de patógenos específicos. Se evaluó la respuesta inmune mucosal en el buche, antes y después del desafío con *Salmonella* Enteritidis. Las ponedoras comerciales y las aves Leghorn blancas libres de patógenos específicos se desafiaron con 10^8 unidades formadoras de colonias por mililitro de *Salmonella* Enteritidis PT13a y *Salmonella* Enteritidis na1RPT13, respectivamente. Desde el día cero (antes del desafío) y hasta el día 25 a 27 post infección, se tomaron muestras de lavados del buche a intervalos semanales y se realizó evaluación bacteriológica para determinar el progreso de la infección con *Salmonella* Enteritidis. Las muestras de lavado de buche se analizaron mediante la prueba de inmunoensayo asociado a enzimas para detectar la presencia de inmunoglobulina A (IgA) específica para lipopolisacáridos de *Salmonella* Enteritidis. A partir del día 34 post-inoculación se evaluó microscópicamente el tejido linfoide de secciones de buche teñidas con hematoxilina y eosina. Utilizando una escala de 0 a 5, las áreas linfoides se calificaron en base a su morfología, tamaño y celularidad. Los valores numéricos de 0 a 5 (de bajo a alto) representan incrementos progresivos en el tamaño y densidad celular del tejido linfoide. Los resultados de los cultivos bacterianos demuestran que el mas alto porcentaje de muestras de lavados de buche positivas a *Salmonella* Enteritidis ocurrieron en los días 5 a 6 y 11 a 12 posteriores a la inoculación. La declinación progresiva en el porcentaje de muestras de lavados de buche positivas a *Salmonella* Enteritidis ocurrió a medida que avanzaba el tiempo post-inoculación; sin embargo, los días 25 a 27 post-inoculación, la *Salmonella* Enteritidis persistió en las muestras provenientes de las gallinas Leghorn blancas libres de patógenos específicos y en tres de las líneas de ponedoras de huevos blancos. Se observó un incremento marcado de la IgA específica para lipopolisacáridos de *Salmonella* Enteritidis en las muestras de lavado de buche obtenidas entre el día 0 y los días 11 y 12 posteriores a la inoculación. La respuesta de IgA especifica para lipopolisacáridos de *Salmonella* Enteritidis permaneció por encima de la línea basal del día 0 a lo largo de todo el experimento. En secciones de buche tomadas 34 días posteriores a la infección, se observaron agregados linfoides con calificaciones bien definidas de 3 a 5. La comparación a los 34 días de las secciones de buche entre las gallinas infectadas y las gallinas control no infectadas, determinaron un incremento de 1.2 a 4 veces en la tasa de tejido linfoide.

Key words: *Salmonella* Enteritidis, chicken, crop, ingluvies, mucosal immune system, gut-associated lymphoid tissue, IgA, enzyme-linked immunosorbent assay

Abbreviations: ARS = Agricultural Research Service; BG+NB = brilliant green agar with novobiocin; BG+NB+NA = brilliant green agar with novobiocin and nalidixic acid; BSL-2 = biosafety level-2; CFU = colony-forming units; ELISA = enzyme-linked immunosorbent assay; H&E = hematoxylin and eosin; IgA = immunoglobulin A; ILF = isolated lymphoid follicle; LPS = lipopolysaccharide; nalR = nalidixic acid resistant; NCSU = North Carolina State University; PBS = phosphate buffered saline; PI = postinfection/postinoculation; PT13 = phage type 13; PT13a = phage type 13a; RV = Rappaport-Vassiliadis; SE = *Salmonella* Enteritidis; SEPRL = Southeast Poultry Research Laboratory; SPF = specific-pathogen-free; ST = *Salmonella* Typhimurium; USDA = United States Department of Agriculture; WL = White Leghorn; XLT4 = xylose lysine tergitol-4 agar

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The chicken crop, a ventral diverticulum of the esophagus, is regarded as a food storage organ in the domestic chicken *Gallus gallus domesticus.* Along with food storage, the crop may act as a depot for commensal and pathogenic organisms (2,5,25,30,32). *Salmonella enterica* subspecies *enterica* serovar Typhimurium (ST) and serovar Enteritidis (SE) are two pathogens that have been cultured from crop contents of broiler- and layer-type chickens (4,6,12,30). Conditions such as stress, feed deprivation, or immunosuppression may alter the crop environment promoting growth of ST and SE within the crop (7,8,13,18,27). ST or SE harbored within the crop may have the potential to spread to other parts of the body, cause subclinical infection, and subsequently lead to establishment of carrier birds in poultry flocks.

The chicken crop may be a potential site along the upper gastrointestinal tract where a mucosal immune response can be produced against *Salmonella* (17,18,28,29). Seo *et al.* (28,29) revealed an IgA humoral immune response within the crop against *Salmonella* Enteritidis (SE) when specific-pathogen-free (SPF) White Leghorn (WL) hens were experimentally challenged with SE via oral gavage. Enzyme-linked immunosorbent assay (ELISA) detected elevated levels of SE-specific IgA in crop lavage samples collected at week 1 postinfection (PI) through week 4 PI (16,17,18,28,29). Microscopic examination by Holt *et al.* (17) of hematoxylin and eosin (H&E) stained crop tissues from post-SE-challenged SPF WL hens showed lymphoid aggregations/lymphoid follicles present in the crop lamina propria. Thus, the crop organ of the chicken may perhaps function as a component of the extensive mucosal immune system.

The ability of ST and SE to reside within and colonize the crop makes this organ an anatomical location worthy of further investigation concerning *Salmonella* pathogenesis. The antigen-specific IgA humoral immune response detected in the chicken crop after SE infection could be of relevance as a diagnostic indicator of enteric disease status and/or useful in identifying prospective oral immunostimulants of the alimentary tract for poultry mucosal vaccines. The crop may also be an organ of importance in the study of gut-associated lymphoid tissue and mucosal immunity. More knowledge about the behavior of ST and SE in relation to the chicken crop and the mucosal immune system is needed so that more effective control measures against infection in poultry can be designed and applied.

SPF WL pullets and SPF WL layer hens have been the primary animal models used to study the mucosal immune response within the chicken crop against SE infection (17,28,29). However, there is a degree of uncertainty regarding the applicability of results obtained from crop-SE experiments using SPF WL chickens to the effects and outcomes produced by SE infection in commercial egg-layer hen strains. Information is lacking about commercial egg-layer strains of chickens in regard to the presence of an IgA humoral response and existence of lymphoid tissue in the crop organ. We sought to determine if SE-antigen-specific IgA response and lymphoid tissue would be observed in the crop organ of SPF WL hens as well as commercial egg-layer strains of chickens post-SE challenge, and to make sure that our previous crop-SE findings were not merely occurrences restricted solely to our line of SPF WL chickens.

Research was undertaken in an effort to determine if data from SE-infected SPF WL hens would be applicable to commercial strains of layer hens in the poultry industry. The purpose of the study was to compare the crop post-SE challenge among eight commercial strains of layer hens and SPF WL chickens. The experiment had three objectives: first was to monitor SE prevalence within the crop at various time points post-SE inoculation; second was to investigate the evolution of a local humoral SE-specific IgA immune response in the crop; and third was to assess the crop for lymphoid tissue presence in SE-challenged hens *vs.* uninfected controls.

MATERIALS AND METHODS

Animals. SPF WL hens used in trial 1 of the study were obtained from flocks maintained by the Southeast Poultry Research Laboratory (SEPRL), USDA-ARS, Athens, Georgia. Hens at approximately 70 weeks of age were housed within individual layer cages located within a climate-controlled biosafety level-2 (BSL-2) building. Birds were allowed access to antibiotic-free feed and water *ad libitum.*

Layer pullets from eight commercial layer strains (five white-egg layer and three brown-egg layer) used in trial 2 were received from the 36th North Carolina Layer Performance and Management Test conducted by the Cooperative Extension Service at North Carolina State University (NCSU) and North Carolina Department of Agriculture and Consumer Services (1). The flock was maintained at the Piedmont Research Station, Salisbury, NC. The eight commercial strains consisted of Lohmann LSL Lite; Dekalb White; Hy-Line W-36; Hy-Line W-98; Bovans White; Bovans Goldline; Bovans Brown; and Hy-Line Brown. At appropriate time points during the rearing period, day of hatch through 16 weeks of age, the Piedmont Research Station personnel routinely vaccinated all pullets against viral diseases: Marek's (herpesvirus), Newcastle (paramyxovirus), fowl pox (poxvirus), and avian encephalomyelitis (picornavirus). The layer pullets at 17 weeks of age were transported from the Piedmont Research Station to the J. Phil Campbell USDA-ARS facility in Watkinsville, Georgia. Birds were grouped by strain and housed within floor pen containment units. Separate containment modules were used for each of the eight strains. Antibiotic-free feed and water was provided *ad libitum.* At 24 weeks of age, birds were moved to a BSL-2 building at SEPRL, USDA-ARS, Athens, Georgia. Birds were arranged according to same strain and placed in individual layer cages (one bird per cage). Free access to antibiotic-free feed and water was provided.

Bacterial strains. SE phage type (PT) 13 and SE PT13a were selected for experimental challenge of layer hens in trial 1 and trial 2, respectively, due to the ability of SE to colonize the reproductive organs of mature laying hens and the identification of phage types 13 and 13a in egg-associated food-borne salmonellosis outbreaks among humans (3,9,10,15,24). Frozen stocks maintained at −70 C of a nalidixic-acid-resistant (nalR) SE PT13 (strain SE89-8312) and SE PT13a (strain 6) were thawed and cultured on nutrient agar at 37 C for 18 hr. Isolated colonies were selected and cultivated in 10 ml tryptic soy broth at 37 C for 18 hr. Broth cultures were then diluted in sterile physiological saline to prepare 10^8 colony-forming units (CFU) /ml inoculum for experimental infection of hens.

Experimental design. *Salmonella*-free status of trial 1 and trial 2 chickens was determined before commencement of the experiment. Feces and crop lavage samples were selectively enriched in Rappaport-Vassiliadis (RV) broth for 24 hr at 37–42 C then plated onto brilliant green agar containing 20 μg/ml novobiocin (BG+NB). *Salmonella* was not detectable.

For trial 1, 20 SPF White Leghorn hens were separated into two groups, experimental and control. Twelve hens made up the experimental group, and each hen was infected *per os* with 1 ml of 10^8 CFU/ml SE nalR PT13. Eight additional SPF WL hens remained uninfected to serve as the control group.

For trial 2, a total of 63 hens were randomly selected from the eight groups of commercial layer strains to serve as experimental and control birds. Five groups were of white-egg-layer–type hens: Lohmann LSL Lite; Dekalb White; Hy-Line W-36; Hy-Line W-98; and Bovans White. Three groups were brown-egg-layer–type hens: Bovans Goldline, Bovans Brown, and Hy-Line Brown. Forty commercial layer strain birds (five hens from each of the eight strains) were experimentally infected *per os* with 1 ml of 10^8 CFU/ml of SE PT13a. Twenty-three birds remained uninfected as controls (one to five birds represented each of the eight strains). In an effort to prevent any perceived bias to a particular

commercial strain, we randomly assigned W1–W5 to the five white-egg layer strains and Br6-Br8 to the three brown-egg layer strains. The W1–W5 and Br6–Br8 designations will be used from this point forward to refer to hens from the eight commercial strains in trial 2.

Crop lavage samples were obtained from trial 1 and trial 2 hens for bacteriological assessment of SE and for ELISA analysis of humoral immune response. Feces were collected from trial 2 hens for bacterial culture to monitor SE fecal shedding. Crop lavage samples were collected from trial 1 hens on the following days: day 0 pre-challenge, day 5 PI, day 12 PI, day 19 PI, and day 27 PI. Trial 2 hens had crop lavage samples and feces collected on day 0 pre-challenge, day 6 PI, day 11 PI, day 18 PI, and day 25 PI. The entire crop organ was harvested at day 34 PI from trial 1 and trial 2 hens for histopathological evaluation of crop tissue. Studies conducted were approved by and in accordance with guidelines of the SEPRL Institutional Animal Care and Use Committee (IACUC).

Crop lavage sample collection. Crop lavages were performed according to the protocol designed by Holt *et al.* (17). The crop lavage devices were fashioned using 10 ml syringes and attached tubing 7–10 inches (17.8–25.4 cm) in length with the distal 1.5 inches (3.8 cm) fenestrated. The lavage devices were aseptically filled with 5 ml of room temperature, sterile glycine flush solution, pH 7.8–8.0 (17,20). Birds were held in an upright, vertical position with neck extended to facilitate insertion of the lavage device tubing into the esophagus. Once the fenestrated portion of tubing was positioned within the crop, the 5 ml glycine solution was slowly infused. Immediately a fluid sample was aspirated back into the syringe, then the crop lavage sample (aspirate) was dispensed from syringe to a sterile collection container. The lavage procedure was repeated per individual bird using a new lavage device and separate sample collection container.

Bacteriological examination. Appropriate techniques and media for isolation of salmonellae were utilized for bacteriological examination of samples collected post-SE infection (31). Trial 1 bacterial culture for SE nalR PT13 was performed using crop lavage samples collected at weekly time points: day 0 pre-challenge, day 5 PI, day 12 PI, day 19 PI, and day 27 PI. A 100 µl portion from each neat crop lavage sample was directly spread plated onto brilliant green agar (BG+NB+NA) containing novobiocin (NB, 20 µg/ml) and nalidixic acid (NA, 20 µg/ml). A 1.0 ml aliquot from each crop lavage sample was added to 9 ml RV broth for selective enrichment. The BG+NB+NA direct spread plates and RV sample tubes were incubated at 37 C overnight. Plates were then assessed for SE colony growth. In instances where direct plating resulted in no growth, the RV-enriched samples were streak plated on BG+NB+NA agar, and streak plates were incubated 24 hr at 37 C, then assessed for typical SE colony growth. Crop lavage samples were recorded as positive if SE was recoverable either by direct plating or after selective enrichment. Negative crop lavage samples were those that had no SE growth detectable.

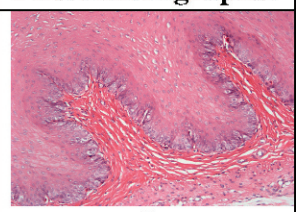
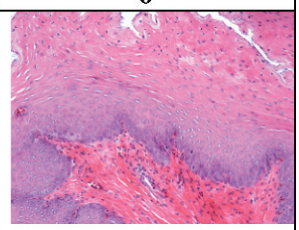
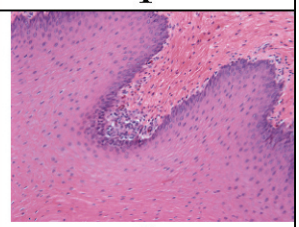
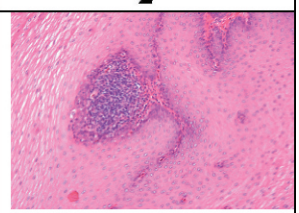
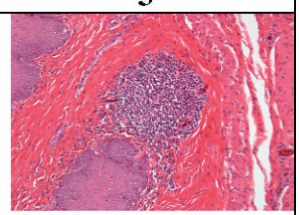
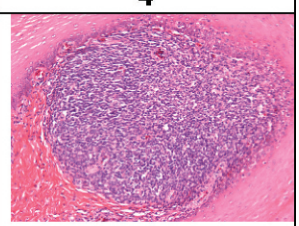
Trial 2 crop lavage samples and feces were cultured for SE PT13a at weekly time points: day 0 pre-challenge, day 6 PI, day 11 PI, day 18 PI, and day 25 PI. A 1.0 ml aliquot from each neat crop lavage sample and a 1.0 gram weight of feces were added to 9 ml RV broth. The RV sample tubes were incubated at 37–42 C overnight. After 24 hr selective enrichment, samples from RV were streak plated onto two media, BG+NB containing novobiocin (NB, 20 ug/ml) and xylose lysine tergitol-4 (XLT4) agar (Beckton Dickinson-Difco, Franklin Lakes, NJ). The BG+NB and XLT4 plates were incubated at 37 C overnight, then examined for bacterial growth and colony morphology indicative of SE. Questionable colonies were further differentiated based on biochemical tests using triple sugar iron agar and modified-lysine iron agar slant tubes, and agglutination reaction with antiserum against *Salmonella* serogroup D_1 somatic antigen (Beckton Dickinson-Difco). Positive samples were those that had SE definitively identified from culture on BG+NB and/or XLT4.

Crop SE-specific IgA ELISA. The crop lavage samples obtained at weekly time points from trial 1 and trial 2 hens were analyzed by ELISA for SE-lipopolysaccharide (LPS)-specific IgA as previously described by Holt *et al.* (15,16,17). A 1.5–2.0 ml portion from each neat crop lavage sample was centrifuged at 10,000 rpm for 5 min to remove feed particulate debris and excess mucous. The supernatant fraction was collected and stored at −20 C. For assay, the frozen crop supernatant samples were thawed, diluted 1:1 with phosphate buffered saline (PBS), containing 0.05% Tween20 (Sigma-Aldrich, St. Louis, MO), then twofold serial dilutions were prepared. Sample dilutions 1:2 to 1:128 were added to flat-bottomed 96-well polystyrene ELISA plates (Corning-Costar Inc., Wilkes Barre, PA) to which SE-LPS antigen (Sigma-Aldrich) at concentration 10 µg/ml had been adsorbed. Additionally, previously defined crop lavage samples with strong and weak SE-LPS-specific IgA antibody responses were run on each assay plate as positive and negative controls, respectively. Plates containing samples and controls were incubated 60 min at room temperature, then washed three times with PBS containing 0.05% Tween20. The next two sequential steps performed were the application of primary antibody with 60-minute incubation time followed by three washes, then addition of an enzyme conjugated secondary antibody with protocol repeated for incubation time and washes. The primary antibody for detection of SE-LPS-specific IgA in crop lavage samples was a monoclonal mouse anti-chicken IgA (Southern Biotech, Birmingham, AL) diluted 1:1000 in PBS containing 0.05% Tween20 and 1% bovine serum albumin. Goat anti-mouse IgG with alkaline phosphatase conjugate (Calbiochem, La Jolla, CA) diluted 1:1000 served as the enzyme conjugated secondary antibody. Diethanolamine buffer with concentration 1 mg/ml p-nitrophenyl-phosphate (Sigma-Aldrich) was applied to plate wells as substrate solution, and the chromogenic reaction proceeded for 15–30 min at room temperature in the dark. Colorimetric analysis with Multiskan Ascent (Thermo Fisher Scientific, Milford, MA) microplate photometer at 405 nm wavelength yielded absorbance/optical density values.

Crop histology and lymphoid tissue scoring. On the final day of the experiment for trial 1 and trial 2, the SE-challenged hens at day 34 PI and negative control hens were euthanatized via carbon dioxide gas inhalation. Immediately post mortem the entire crop with 1.0 inch (2.54 cm) of attached esophagus was excised. Tissues were fixed intact in 10% neutral buffered formalin for 24 hr, and the crops were then sectioned. Transverse cuts were made at the midbody region of the crop. The plane of the cut passed through the entirety of the hollow-bodied crop organ to produce ring-shaped circular sections. Crop tissue sections were routinely processed, paraffin embedded, microtome cut, and affixed to glass slides. Tissue slides were stained with H&E, then examined via light microscopy. The H&E crop tissues were evaluated full circle for the presence of inflammatory changes and lymphoid aggregations in the epithelium and lamina propria. All apparent sites of lymphocytic-lymphoid cell infiltrates and/or isolated lymphoid follicles (ILFs) were graded as score 0 to 5, and the number of lymphoid areas observed for each score category was recorded. An ocular grid reticle (Fisher Scientific, Suwanee, GA) was used to make the score 0 to 5 determinations and perform counts at 200× magnification. Criteria used to establish the score 0 to 5 grade scale were morphological character, size, and cellular density of a lymphoid site. The score 0 to 5 numerical increase represented lymphoid tissue that progressively increased in structural detail, size, and cellularity (Table 1).

Statistical analyses. Statistical analyses were performed using GraphPad Instat (GraphPad Software, Inc., San Diego, CA). Analysis of variance (ANOVA) with Tukey's multiple comparison test procedure were conducted on bacterial culture results and ELISA data for trial 1 and trial 2 hen strains to determine statistically significant differences ($P < 0.05$) among the post-SE infection time points. ANOVA with Dunnett's multiple comparison test procedures were conducted on bacterial culture results and ELISA data to determine statistically significant differences ($P < 0.05$) between pre-challenge day 0 control and post-SE-challenge time points for trial 1 and trial 2 hen strains. Unpaired *t*-tests were performed on crop lymphoid tissue score data to assess whether or not the uninfected control group and day 34 PI SE-challenged group differed significantly ($P < 0.05$) for each of the hen strains. The humoral response within the crop of SPF WL hens and commercial layer hen strains (W1–W5, Br6–Br8) was compared for statistically significant differences ($P < 0.05$) by ANOVA and Tukey's multiple comparison posttest procedure. Significant differences ($P <$

Table 1. Interpretive illustrations for the score 0 to 5 crop lymphoid tissue grade scale.

Score 0 to 5 Classification of Lymphoid Tissue Areas in Chicken Crop			
SCORE	[a b] **Size Criteria:**	[a] **Morphologic/Cellular Criteria:**	**Photomicrographs:**
0	0 to < 1 grid square	Absence of inflammatory cell infiltrate. (Lymphocytic-lymphoid cells within normal limits for resident population in epithelium and lamina propria of gastrointestinal tract.)	 **0**
1	1 x 1 grid square; ~50 µm diameter	Locally diffuse area of lymphocytic-lymphoid cell infiltration; low cellular density.	 **1**
2	2 x 2 grid squares; >50 to 100 µm diameter	Focal coalescence of lymphocytic-lymphoid cells; low to slightly mild-moderate cellular density.	 **2**
3	3 x 3 grid squares; >100 to 150 µm diameter	Defined focal aggregation of lymphocytic-lymphoid cells; moderate to heightened cellularity.	 **3**
4	4 x 4 grid squares; >150 to 200 µm diameter	Well-demarcated, circular-ellipsoidal focal area of lymphocytic-lymphoid cells; hypercellular; organized aggregation.	 **4**
5	5 x 5 grid squares or larger; >200 to 250+µm diameter	Sharply defined, focal area of lymphocytic-lymphoid cells; extreme hypercellularity; organized aggregation; locally expansive, may extend from lamina propria into basal epithelium.	 **5**

[a] Light microscopy examinations, morphometric analyses and score 0 to 5 grading of lymphoid tissue sites were conducted at 200X magnification with aid of ocular grid reticle (1 grid square=50 µm length x 50 µm width).

[b] Size of lymphoid area was established based on measurements of the diameter at widest point across the lymphoid structure and/or the number of grid squares (length x width) that defined entire area of involvement.

Table 2. Recovery of SE from crop lavage samples and feces of SPF WL hens and eight commercial layer strains of chickens (white-egg layer, W1–W5, and brown-egg layer, Br6–Br8).

	Salmonella Enteritidis positive/total [A]									
	Day 0		5–6 days		11–12 days		18–19 days		25–27 days	
	Pre-infection		Post-infection		Post-infection		Post-infection		Post-infection	
Hen strain	Crop	Feces	Crop	Feces	Crop	Feces	Crop	Feces	Crop	Feces
SPF WL	0/12[a]	0/12[a]	12/12[a]	No data	5/12[a]	No data	6/12[a]	No data	3/12[a]	No data
W1	0/5[a]	0/5[a]	4/5[a,b]	4/5[a]	2/5[a]	1/5[a]	3/5[a]	3/5[a]	1/5[a]	1/5[a]
W2	0/5[a]	0/5[a]	4/5[a,b]	5/5[a]	4/5[a]	3/5[a]	1/5[a]	1/5[a]	1/5[a]	1/5[a]
W3	0/5[a]	0/5[a]	3/5[a,b]	5/5[a]	5/5[a]	2/5[a]	2/5[a]	1/5[a]	1/5[a]	0/5[a]
W4	0/5[a]	0/5[a]	5/5[a]	5/5[a]	5/5[a]	3/5[a]	4/5[a]	1/5[a]	0/5[a]	0/5[a]
W5	0/5[a]	0/5[a]	5/5[a]	4/5[a]	5/5[a]	1/5[a]	2/5[a]	2/5[a]	0/5[a]	0/5[a]
Br6	0/5[a]	0/5[a]	2/5[a,b]	5/5[a]	2/5[a]	3/5[a]	1/5[a]	2/5[a]	0/5[a]	0/5[a]
Br7	0/5[a]	0/5[a]	4/5[a,b]	5/5[a]	4/5[a]	4/5[a]	2/5[a]	4/5[a]	0/5[a]	4/5[a]
Br8	0/5[a]	0/5[a]	1/5[b]	5/5[a]	1/5[a]	4/5[a]	0/5[a]	5/5[a]	0/5[a]	2/5[a]
Total	0/52[a]	0/52[a]	40/52[b]	38/40[b]	33/52[b]	21/40[b]	21/52[b]	19/40[b]	6/52[a]	8/40[b]

[A]Values within each column that have unlike lowercase letters are significantly different ($P < 0.05$). Totals in bottom row with dissimilar lowercase letters are significantly different ($P < 0.05$) from day 0 pre-infection control.

0.05) in the mean number of crop ILFs observed at day 34 post-SE infection between SPF WL and commercial layer hen strains were determined by ANOVA with Tukey's multiple comparison post-test procedure.

RESULTS

Bacterial culture. Table 2 represents the temporal kinetics of SE prevalence in weekly samples cultured from SPF WL hens and each of the eight commercial egg-layer strains. The prevalence of SE from crop lavage samples among trial 1 SPF WL hens was 0% at day 0 pre-challenge, then 100% day 5 PI, 42% day 12 PI, 50% day 19 PI, and 25% day 27 PI. For the trial 2 commercial layer hen strains (W1–W5, Br6–Br8), no SE was recoverable from crop lavage samples or feces cultured at day 0 pre-challenge. The percentage of SE positive samples among the five white-egg layer (W1–W5) strains at day 6 PI ranged from 60% to 100% (mean 84%) crop and 80% to 100% (mean 92%) feces. Among the three brown-egg layer (Br6–Br8) strains at day 6 PI, SE positive samples ranged from 20% to 80% (mean 47%) crop and were 100% from feces. The percentage of SE positive samples at day 11 PI among the five white-egg layer (W1–W5) strains ranged from 40% to 100% (mean 84%) crop and 20% to 60% (mean 40%) feces. At day 11 PI the three brown-egg layer (Br6–Br8) strains ranged from 20% to 80% (mean 47%) crop and 60% to 80% (mean 73%) feces SE culture positive. Detection of SE prevalence at day 18 PI ranged from 20% to 80% (mean 48%) crop and 20% to 60% (mean 32%) feces among W1–W5 strains. At day 18 PI, SE prevalence among Br6–Br8 strains was 0% to 40% (mean 20%) crop and 40% to 100% (mean 73%) feces. The SE culture positive samples at day 25 PI among the W1–W5 strains ranged from 0% to 20% (mean 12%) crop and 0% to 20% (mean 8%) feces. The Br6–Br8 strains had 0% SE recovered from crop lavage samples at day 25 PI, and fecal shedding of SE was 0%, 80% and 40% (mean 40%) among Br6, Br7, and Br8 strains, respectively. Comparison of trial 1 (SPF WL) and trial 2 (W1–W5, Br6–Br8) culture results from crop lavage samples and feces revealed that the average percentages of SE positive samples were highest at the earlier time points following SE challenge (Table 2). As time lengthened post-SE challenge, then a progressive decline in percentage of SE positive samples occurred. However, SE infection persisted within the crop through days 25–27 PI for SPF WL, W1, W2, and W3 hen strains. Shedding of SE in feces continued through day 25 PI for hen strains W1, W2, Br7, and Br8.

Crop SE-LPS-specific IgA. ELISA detected an increased SE-lipopolysaccharide (LPS)-specific IgA response in crop lavage supernatant samples from trial 1 and trial 2 post-SE-challenged hens. Crop SE-LPS-specific IgA responses over time for SPF WL hens in trial 1 and each of eight commercial strains W1–W5, Br6–Br8 in trial 2 are depicted in Figure 1. The humoral immune response detected post-SE-challenge within the crop of trial 1 and trial 2 hens was of the same relative pattern. An IgA humoral immune response against SE-LPS appeared within the crop following SE oral challenge, and SE-LPS IgA remained elevated for duration of the experiment. Prior to challenge at day 0, the SE-LPS-specific IgA levels within the crop were minimal. A slight increase in SE-LPS-specific IgA response was observed between day 0 and day 5–6 PI. By 11–12 days PI, the SE-LPS-specific IgA response in the crop increased dramatically. Statistically significant differences ($P < 0.05$) from day 0 were observed for crop SE-LPS-specific IgA at 11–12 days PI (Fig. 1). The SE-LPS-specific IgA response remained elevated greater than day 0 baseline at days 18–19 PI and days 25–27 PI. The humoral response within the crop post-SE infection was similar for the SPF WL hens and the eight commercial egg-layer hen strains (Fig. 1). There were no statistically significant differences ($P > 0.05$) between the egg-layer hen strains (SPF WL hens *vs.* commercial hen strains) at the post-SE infection time points, with the exception of SPFWL *vs.* W3 at day 25–27 PI, as determined by statistical analysis using ANOVA and Tukey's multiple comparison post-test.

Crop histology and lymphoid tissue scores. Illustrations of the various degrees of lymphoid tissue and/or lymphocytic infiltration observed in the crop, and the criteria used for establishment of the score 0 to 5 grade classifications, are presented in Table 1. The ascending 0 to 5 numerical score scale corresponded to lymphoid tissue that progressively increased in size, structural detail, and cellular density. A score of 3 to 5 denoted a well-demarcated, organized lymphoid-lymphocytic aggregation and/or individual lymphoid follicle of moderate to heightened cellularity and of increased size diameter. We felt that the score 3 to 5 lymphoid tissue sites within the crop potentially reflected areas of enhanced antigenic stimulation and induction of an immune system inflammatory response (11,19,22,23). Thus, crop lymphoid tissue sites in the score

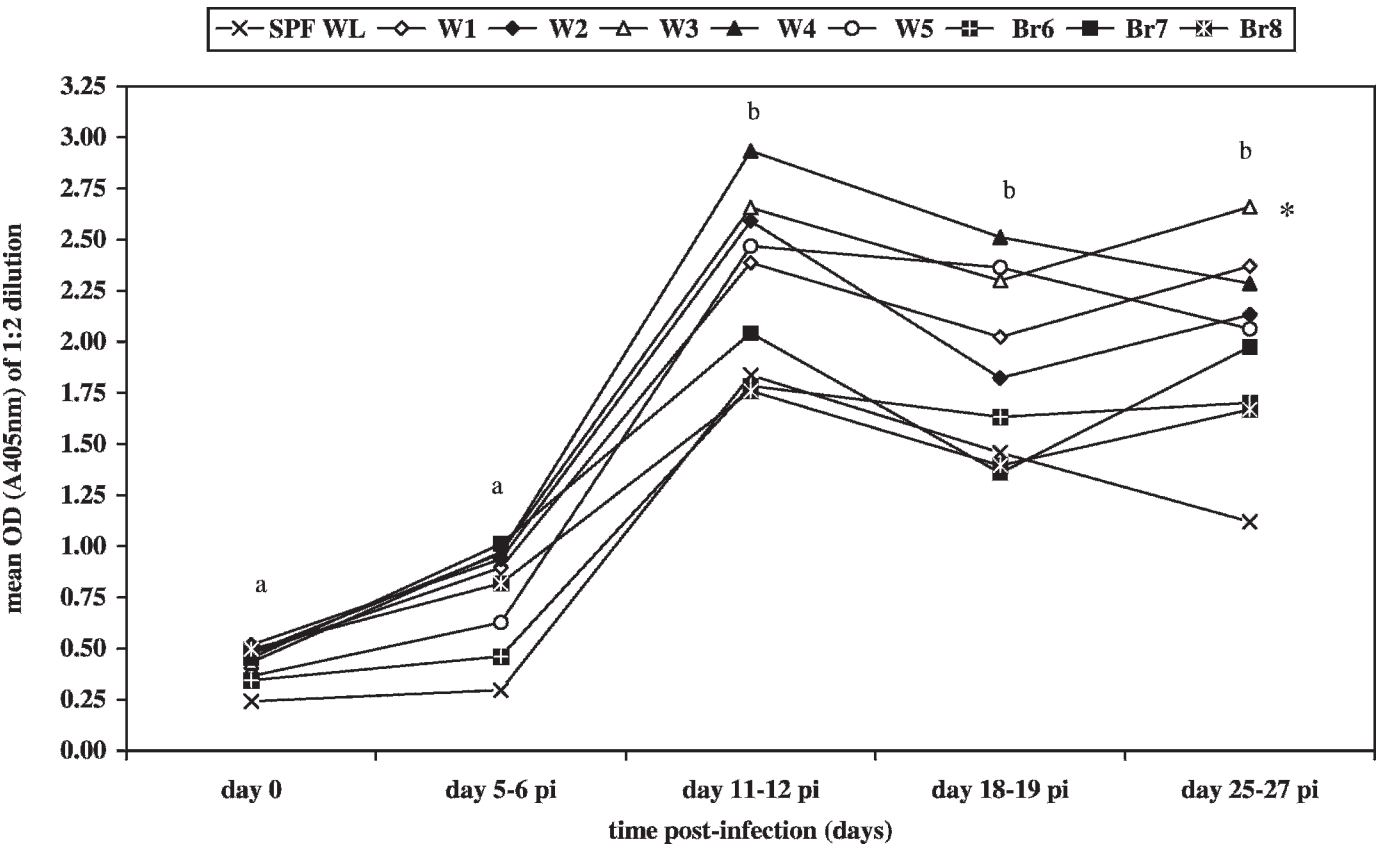


Fig. 1. ELISA analyses of SE-LPS-specific IgA in crop lavage samples from SPF WL hens and eight commercial strains (W1-W5, Br6-Br8). Mean OD values are representative of SE-LPS-specific IgA detected in 1:2 dilution crop lavage supernatant samples at prechallenge (Day 0) and post-SE infection. Dissimilar lowercase letters indicate significant differences ($P < 0.05$) from Day 0 preinfection control. An asterisk represents a statistical difference ($P < 0.05$) between OD values for W3 and SPF WL at day 25–27 PI time point.

3 to 5 range were those selected to make comparisons among the day 34 PI SE-challenged hens and uninfected control hens. Table 3 shows the trial 1 and trial 2 day 34 post-SE-challenged groups and control groups: semiquantitative count distribution for crop lymphoid areas in the score categories 3, 4, and 5; total number count of crop lymphoid areas within score 3–5 range; calculated mean (average) number of the score 3–5 range crop lymphoid tissue areas; and ratio of SE day 34 PI to control crop lymphoid tissue. The H&E crop sections from uninfected control groups in trial 1 and trial 2 contained lymphoid tissue areas that were judged as score 3, 4, and 5. The total number of score 3–5 range lymphoid areas in the control groups ranked from a low count of 5 to a high of 15. Examination of H&E crop sections from day 34 post-SE-challenged hen groups found an increased number of score 3 to 5 lymphoid tissue areas in the SE-challenged compared to uninfected controls. Crop sections from day 34 PI SE-challenged groups had total number counts ranked at 18 low to 36 high for lymphoid areas within score range 3–5. Figure 2 compares the mean number of score 3–5 range crop lymphoid tissue areas assessed for the day 34 PI SE-challenged group *vs.* uninfected control group from each hen strain. Averages of crop lymphoid tissue among the day 34 PI SE-challenged groups were greater than the uninfected control groups for trial 1 SPF WL hens and each of the eight commercial strains in trial 2. The SPF WL trial 1 hens and trial 2 commercial W1–W5, Br6–Br8 hen strains had 1.2 to 4.0-fold increased ratio of day 34 PI SE-challenged crop lymphoid tissue compared to uninfected controls (Table 3). An overall increase of score 3–5 range ILFs/ lymphoid tissue areas in crop sections from day 34 PI SE-challenged groups as compared to control groups was seen for trial 1 SPF WL hens and the trial 2 hen strains W1–W5, Br6–Br8. ANOVA and Tukey's post-test determined no statistically significant differences ($P > 0.05$) in the mean number of crop lymphoid tissue areas assessed at day 34 post-SE infection between SPF WL hens and the eight commercial egg-layer hen strains.

DISCUSSION

Experimental SE challenge did induce a similar or comparable immune response in the crop post-SE infection among the SPF WL hens and the eight commercial egg-layer strains (W1–W5, Br6–Br8). Results were similar in regards to kinetics of SE prevalence (Table 2), humoral SE-LPS IgA response (Figure 1), and lymphoid cellular infiltrate presence (Fig. 2) within the crop. Trends observed for the SPF WL and commercial layer-hen strains were higher percentages of SE culture positive crop lavage and fecal samples detected at early time points post-challenge with gradual decline in percentage of SE detected over time post-infection (Table 2); initial crop lavage samples with minimal to low SE-LPS-specific IgA then elevated IgA antibody responses specific against SE-LPS antigen at 11–12 days post-SE infection (PI) to 25–27 days PI (Fig. 1); and increased lymphoid tissue evaluated in crop sections from SE-challenged hens *vs.* uninfected controls (Table 3; Fig. 2). Data obtained from this study and other SE-crop immunity studies conducted by Seo *et al.* (28,29) and Holt *et al.* (17) that involved the

Table 3. Numerical data for lymphoid tissue areas graded as score 3–5 that were observed in H&E stained crop sections from *Salmonella* Enteritidis challenged (SE day 34 PI) group and uninfected (control) group per each of the different hen strains.

		Crop lymphoid tissue counts					Lymphoid tissue ratios
Strain	Group[A]	Score 3	Score 4	Score 5	Total no.	Mean no.[B]	(SE day 34 PI /control)
SPF WL							
	SE day 34 PI ($n = 12$)	16	11	9	36	3.0	3.3
	Control ($n = 8$)	1	5	1	7	0.9	
W1							
	SE day 34 PI ($n = 5$)	11	6	14	31	6.2	1.2
	Control ($n = 3$)	4	4	7	15	5.0	
W2							
	SE day 34 PI ($n = 5$)	10	8	5	23	4.6	1.3
	Control ($n = 4$)	6	2	6	14	3.5	
W3							
	SE day 34 PI ($n = 5$)	5	7	6	18	3.6	1.4
	Control ($n = 2$)	2	1	2	5	2.5	
W4							
	SE day 34 PI ($n = 5$)	15	8	11	34	6.8	4.0
	Control ($n = 3$)	2	1	2	5	1.7	
W5							
	SE day 34 PI ($n = 5$)	8	7	7	22	4.4	1.7
	Control ($n = 5$)	2	3	8	13	2.6	
Br6							
	SE day 34 PI ($n = 5$)	12	3	15	30	6.0	1.5
	Control ($n = 2$)	2	2	4	8	4.0	
Br7							
	SE day 34 PI ($n = 5$)	9	6	8	23	4.6	2.3
	Control ($n = 1$)	1	1	0	7	2.0	
Br8							
	SE day 34 PI ($n = 5$)	9	6	11	26	5.2	1.6
	Control ($n = 3$)	5	2	3	10	3.3	

[A] n = number of crops sampled.
[B] No significant differences ($P > 0.05$) were determined.

use of specific pathogen free SPF WL chickens as the animal model do appear applicable to commercial egg-layer strains of chickens.

All of the SE-challenged hen groups (SPF WL, W1–W5, and Br6–Br8) showed elevated IgA against SE-LPS antigen in the crop lavage samples that were collected at days 5–6 PI through days 25–27 PI (Fig. 1). The increased SE-LPS-specific IgA response detected in crop lavage samples at days 5–6 PI and days 11–12 PI correlated with bacteriological examination time points in which the highest percentages of SE culture positive crop lavage samples were observed (Table 2). The concomitant findings of viable SE, SE-LPS-specific IgA, and lymphoid tissue within the crop organ seem to support the ability of the crop to actively produce a humoral immune response when exposed to the pathogen. However, it is not conclusive that the generation of SE-specific IgA occurred locally within the crop; therefore future studies are planned to try to identify and recover SE-antigen specific B-lymphocytes from the crop of SE-infected chickens.

The prevalence of SE within the crop did decline over time post-SE inoculation, which may have been a result of an enhanced humoral immune response. However, the humoral IgA response found within the crop may not have been capable of providing complete protection against SE colonization nor have the ability to fully combat SE infection of the alimentary tract, as complete clearance of SE from the crop and feces of all the experimentally infected hens did not occur. At days 25–27 PI, *Salmonella* Enteritidis survived and persisted within the crop (25% SPF WL; 20% W1; 20% W2; 20% W3) and feces (20% W1; 20% W2; 80% Br7; 40% Br8) of a few of the experimentally challenged hens despite the moderate to heightened SE-specific IgA response detected within the crop (Table 2; Fig. 1). A slightly more similar outcome in regard to SE prevalence within the crop of SPF WL and commercial white-egg layer strains, as compared to SPF WL and brown-egg layer hen strains, might perhaps be linked to genetics. The SPF WL hens and commercial white-egg layer hen strains used for this study could have perhaps had a close genetic relatedness. A shared ancestral linkage between the SPF WL and commercial white-egg layer strains to that of the WL layer might have perhaps been a factor that influenced mucosal immune response and persistence/clearance of the SE pathogen. A genetic divergence of the brown-egg layer strains away from WL to perhaps incorporation of broiler stock lineage might have contributed to variation of SE persistence in the crop between the SPF WL and commercial brown-egg layer strains. Genetic variation of poultry breeds can influence immunocompetence and resistance to infection (21,26).

In the present study SPF WL chickens and commercial egg-layer hens exhibited a comparable SE-LPS-specific IgA response (Fig. 1) and presence of lymphoid tissue (Fig. 2) within the crop; thus we feel confident to advance with mucosal immunology experiments using SPF WL chickens as the animal model. Additional SE infection studies over an extended time period will be performed to assess long-term temporal dynamics of the humoral immune response against SE within the crop. Future research will be conducted to characterize the cellular nature of lymphoid aggregations observed in the crop post-SE infection. Flow cytometry and/or immunohistochemical staining methods will be employed in an effort to identify the cellular repertoire of the crop, and determine if cellular populations (i.e., B-lymphocytes and plasma cells) exist that are capable of generating the mucosal humoral immune response locally within the crop organ.

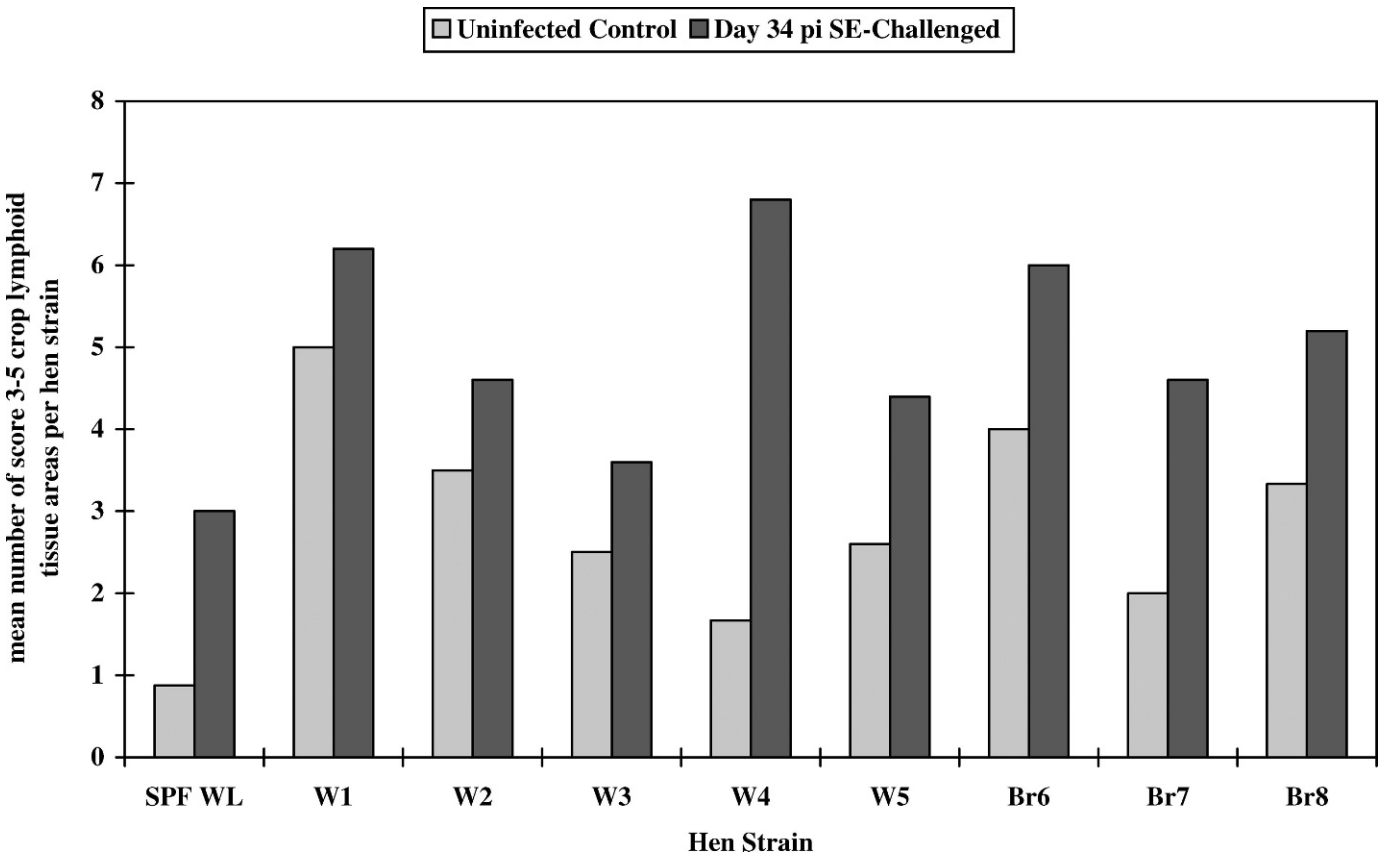


Fig. 2. Average number of score 3–5 range lymphoid tissue areas counted in H&E-stained crop sections prepared from uninfected control group and Day 34 pi SE-challenged group for each of the hen strains (SPF WL = specific pathogen free White Leghorn; W1–W5 = commercial white-egg layers; Br6–Br8 = commercial brown-egg layers). No significant differences ($P > 0.05$) were determined for lymphoid tissue observed at Day 34 pi from the hen strains.

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